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(71) Applicant *(for all designated States except US)*: **IVAX CORPORATION** [US/US]; 4400 Biscayne Boulevard, Miami, FL 33137 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **JEKKEL, Antonia** [HU/HU]; Damjanich u. 38, H-1071 Budapest (HU). **AMBRUS, Gabor** [HU/HU]; Csalan u. 45/b, H-1025 Budapest (HU). **ILKOY, Eva** [HU/HU]; Munkacsy u. 37, H-1046 Budapest (HU). **HORVATH, Ildiko** [HU/HU]; Boloni Gy. u. 9, H-1021 Budapest (HU). **KONYA, Attila** [HU/HU]; Lovas I. u. 13, H-5000 Szolnok (HU). **SZABO, Istvan, Mihaly** [HU/HU]; Bertalan L. u. 11, H-1111 Budapest (HU). **NAGY, Zsuzsanna** [HU/HU]; Eotvos u. 12, H-1181 Budapest (HU). **HORVATH, Gyula** [HU/HU]; Kigyo u. 56, H-1052 Budapest (HU). **MOZES, Julianna** [HU/HU]; Borso u. 56, H-1173 Budapest (HU). **BARTA, Istvan** [HU/HU]; Kassai u. 10, H-1142 Budapest (HU). **SOMOGYI, Gyorgy** [HU/HU]; Jerney u. 70, H-1148 Budapest (HU). **SALAT, Janos** [HU/HU]; Veresegyhaz u. 21, H-1151 Budapest (HU). **BOROS, Sandor** [HU/HU]; Nyar u. 86, H-2134 Szod (HU).

(74) Agents: **BRAINARD, Charles, R.** et al.; Kenyon & Kenyon, One Broadway, New York, NY 10004 (US).

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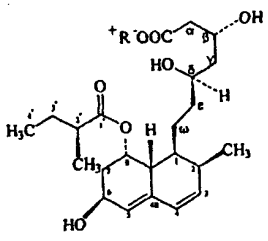
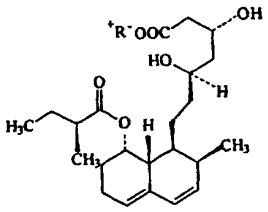
(54) Title: MICROBIAL PROCESS FOR PREPARING PRAVASTATIN



(I)



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(57) Abstract: The present invention relates to a new microbial process for the preparation of the compound of formula (I) from a compound of general formula (II) wherein R^+ stands for an alkali metal or ammonium ion, by the submerged cultivation of the strain able to 6β-hydroxylate a compound of formula (II) in aerobic fermentation and by the separation and purification of the product of formula (I) formed in the course of the bioconversion, which comprises cultivating a strain of the genera *Micromonospora* able to 6β-hydroxylate a compound of the general formula (II), wherein R^+ is as defined above, on a nutrient medium containing assimilable carbon and nitrogen sources and mineral salts at 25-32 °C, thereafter feeding the substrate to be transformed into the developed culture, then fermenting the substrate until the end of bioconversion, then separating the compound of formula (I) from the culture broth and, if desired, purifying the same.

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MICROBIAL PROCESS FOR PREPARING PRAVASTATIN

FIELD OF THE INVENTION

The present invention relates to microbial processes for the preparation of pravastatin.

BACKGROUND OF THE INVENTION

Hypercholoesterolemia has been recognized as a major risk factor for atherosclerotic disease, specifically for coronary heart disease. Biosynthesis of cholesterol is a major contributing factor to hypercholesterolemia. HMG-CoA reductase catalyzes the conversion of HMG-CoA to mevalonate in the rate determining step in the biosynthesis of cholesterol. During the past two decades, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase EC. 1.1.1.34) has been extensively studied. Mevinolin and related compounds biosynthesized by different fungal species have been found to be competitive inhibitors of this enzyme [Endo, A. et al., J. Antibiotics 29, 1346-1348 (1976): Endo. A. et al., FEBS Lett. 72, 323-326 (1976): Kuo, C.H. et al., J. Org. Chem. 48, 1991-1998 (1983)].

Pravastatin is a member of this family of HMG-CoA reductase inhibitors, along with compactin, lovastatin, simvastatin, fluvastatin and atorvastatin. Pravastatin was first isolated as a minor canine metabolite of compactin (Tanaka, M. et al., unpublished) in the course of metabolic studies of compactin [Aral, M. et al., Sankyo Kenkyusho Nempo, 40, 1-38 (1988)].

Tissue selectivity is a unique characteristic of pravastatin. Pravastatin selectively inhibits cholesterol synthesis in the liver and small intestine but only weakly inhibits cholesterol synthesis in other organs. Koga, T. et al. *Biochim. Biophys. Acta*, **1990**, *1045*, 115-120. Pravastatin has an advantage of lower toxicity than the other HMG-CoA reductase inhibitors.

It has been reported that compactin can be converted to pravastatin by microbial hydroxylation using various genera of fungi as well as bacteria belonging to the genera *Nocardia,* of the group Actinomycetes; the genera *Actinomudura*, of the group Maduromycetes and the genera *Streptomyces roseochromogenes* and *Streptomyces carbophilus*, among other species of the group *Steptomyces* (U.S. Patent No. 5, 179,013, U.S. Patent No. 4, 448,979, U.S. Patent No. 4,346,227, U.S. Patent No. 4,537,859, Japanese Patent No. 58-10572).

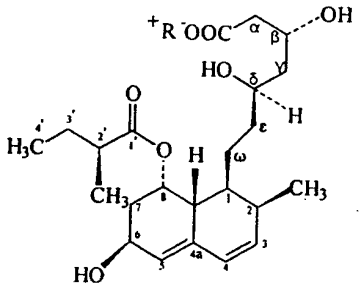
A problem is encountered with the use of fungi for the production of pravastatin. Fungi generally do not tolerate high loads of compactin added in the culture medium, presumably due to the antifungal activity of compactin [Serizawa, N. et al., J. Antibiotics 36, 887-891 (1983)].

The cytochrome P450 system has been shown to be required for the hydroxylation of compactin to pravastatin by *Streptomyces carbophilus* bacteria. [Matsuoka, T. et al., Eur. J. Biochem. 184, 707-713 (1989)]. A problem with the use of the cytochrome P450 system is that recombinant DNA manipulations of it are difficult because it is a complex of proteins rather than a single protein.

There is a need for an improved microbial process for preparing pravastatin that can tolerate high concentrations of compactin and produce pravastatin in high yield and at high concentration in the fermentation broth.

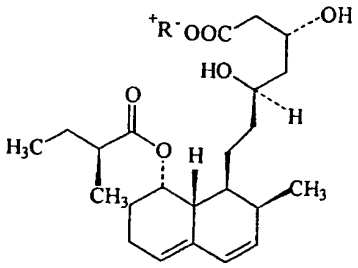
SUMMARY OF THE INVENTION

The present invention provides a new microbial process for the preparation of pravastatin. More particularly, this invention provides a microbial process for the preparation of pravastatin of formula (I)



(I)

from a compound of the general formula (II)



(II)

wherein R^+ stands for an alkali metal or ammonium ion, with a prokaryote from genus *Micromonospora* of the Actinoplanetes group able to hydroxylate a compound of the general formula (II) at the 6β position.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a new microbial process for the preparation of pravastatin.

The present invention is the culmination of an investigation undertaken to find a microorganism that would produce pravastatin at higher concentrations and under more advantageous conditions than has been possible with known microbial systems. Over 6,000 actinomycete strains were screened. Of these, only ten microorganisms were found to be capable of hydroxylating the sodium salt of compactin to produce pravastatin. In particular, the following species had this capacity: *Streptomyces violaceus* No. 1/43 (Kämpfer et al. 1991), *Streptomyces rochei* No 1/41 (Berger et al. 1989), *Streptomyces resistomycificus* No. 1/44 (Lindenbein 1952), *Streptomyces lanatus* (Frommer 1959), *Streptomyces* sp. No. 1/28, *Micromonospora* sp. No. IDR-P_3, *Micromonospora purpurea* No. IDR-P_4 (Luedemann and Brodsky 1964), *Micromonospora megalormicea ssp. nigra* No. IDR-P_6 (Weinstein et al 1969), *Micromonospora rosaria* No. IDR-P_7 (Horan and Brodsky 1986). Since it was not previously known that species of the *Micromonospora* genus were able to convert salts of the acid form of compactin into pravastatin, we undertook a detailed study the *Micromonospora* species that screened positive.

Micromonospora is a genus belonging to the actinomycetes taxonomic group of bacteria. Within the order *Actinomycetales* and the suprageneric group of *Actinoplanetes*, the genus *Micromonospora* has been shown to be more closely related to sporangia-forming actinomycetes, such as *Actinoplanes* and *Dactylosporangium*, and sharply distinct from other monosporic genera such as *Thermomonospora* and

Thermoactinomyces, with which it was has been associated. The genera of *Actinoplanetes* have similar chemotaxonomic characters and nucleic acid affinities. They are Gram-positive, non-acid fast organisms growing with nonfragmenting, branched and septate hyphae of 0.2-1.6 μm in diameter. Aerial mycelium is rarely developed or only sparse. Genus Micromonospora Ørskov, 1923.

Micromonospora chalcea (Foulerton, 1905) form well-developed, branched, septate mycelium averaging 0.5 μm in diameter. Nonmotile spores are formed singly, sessile, or on short or long sporophores that often occur in branched clusters. Sporophore development is monopodial or in some cases sympodial. Aerial mycelium is absent or in some cultures appears irregularly as a restricted white or grayish bloom. Cell walls contain meso-diaminopimelic acid and/or its 3-hydroxy derivative and glycine. Xylose and arabinose are present in cell hydrolysates. Characteristic phospholipids are phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides. *Micromonospora chalcea* are aerobic to microaerobic and are chemoorganotrophic. They are sensitive to pH below 6.0. Growth occurs normally between 20°C and 40°C but not above about 50°C. Ørskov, 1923.

It has been observed that several significantly different species of the genus *Micromonospora* are able to hydroxylate compactin at the 6β-position and, thus, it appears that the ability to hydroxylate compactin at the 6β-position is widely shared by species of genus *Micromonospora*. The *Micromonospora* of the present invention include wild type and mutant strains that are capable of converting a compactin substrate to pravastatin. Preferred *Micromonospora* used to further describe certain preferred embodiments of the invention and to illustrate it with specific examples were selected for

their high hydroxylating capacity, which can exceed about 90% at 0.1 g/liter concentration of compactin acid sodium salt. The following strains of *Micromonospora* were deposited on April 13, 1999 at the National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary under the number NCAIM P (B) 001271 of *Micromonospora purpurea* IDR-P_4; NCAIM P (B) 001272, *Micromonospora echinospora ssp. echinospora* IDR-P_6; NCAIM P (B) 001273, *Micromonospora meg alomicea ssp. nigra* IDR-P_6; and NCAIM P (B) 001274 of *Micromonospora rosaria* IDR-P_7.

An isolated *Micromonospora* species, numbered IDR-P_3, was deposited on October 13, 1998 at the National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary under the number NCAIM P (B) 001268. Strain No. IDR-P_3 of *Micromonospora sp.* was isolated from a mud sample of Lake Balaton, Hungary. In addition to producing pravastatin from compactin sodium salt in high concentration under conditions suitable to large scale fermentation, this species biosynthesizes only minor amounts of other structurally related compounds. Thus this species is very well adapted for the industrial production of pravastatin.

The taxonomic features of the cultures of *Micromonospora* IDR-P_3 are summarized as follows.

Micromorphological properties: Substrate mycelium is composed of well developed, more curved than straight, branching filaments. In slide cultures, the monopodial system of branching hyphae (sporophores) may be observed. Spores are single, spherical, approximately 1.8 μm in diameter and are dispersed evenly on hyphal filaments. Spores are either sessile or on the end of short sporophores. In broth cultures, spores were not

observed on sporulating hyphae, possibly because of the mature spores are released rapidly into the medium.

Cultural-morphological properties:

Czepak-sucrose agar: Medium growth, the colonies are of reddish color covered by point-like black sporulating areas.

Glucose-asparagine agar: The growth was recorded as point-like and elevated, reddish-brown or black colonies. Reddish diffusible pigment.

Nutrient agar: Fair growth, elevated, reddish-brown or black colonies. Reddish-brown exopigment in the medium.

Yeast extract-malt extract agar (ISP Med. 2): Well developed, elevated and wrinkled, brown colonies, covered partly with black sporulating areas or with "pseudo-aerial mycelium" appearing as a restricted whitish or greyish bloom. Brownish or brownish-red soluble pigment.

Inorganic salts-starch agar (ISP Med. 4): Medium growth of reddish-brown elevated and wrinkled colonies. Light reddish soluble pigment.

Glycerol-asperagine agar (ISP Med. 5): Growth only in traces, off-white or light orange colored, flat colonies, light rose soluble pigment.

On some media observing soluble pigment has a particular indicator-character: being yellow in the acid pH-range and in the basic pH-range slightly turns into dark shade of reddish color.

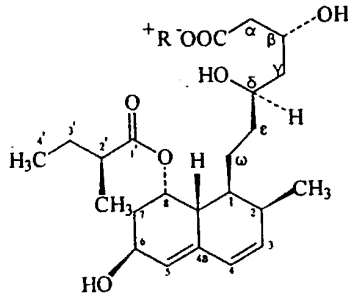
Carbon source utilization: Good growth on and positive utilization of L-arabinose, D-cellobiose, D-fructose, D-glucose, lactose, D-maltose, D-mannitol, D-mannose, α-methyl-D-glucoside, L-rhamnose, D-ribose, D-sucrose, D-trehalose and D-xylose. Adonitol,

dulcitol, myo-inositol, inulin, D-melezitose, D-raffinose are not utilized. Growth with D-galactose, glycerol, D-melibiose and D-salicin was slightly better than on the negative control medium.

Nitrogen source utilization: Good growth with yeast extract and NZ-Amine, no utilization of L-asparagine, L-glutamic acid, NH_4NO_3 and $NaNO_3$.

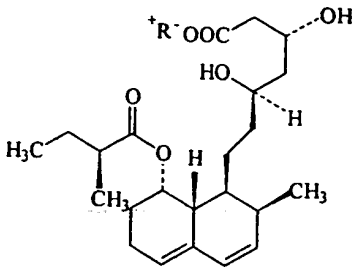
Other physiological-biochemical properties: Cellulose and starch are hydrolyzed, milk is digested strongly. Nitrate reduction test is negative. No growth on potato slices without calcium carbonate (pH 5.8-6.0).

A preferred form of the invention, base upon our studies of the *Micromonospora* strains deposited with the National Collection of Agricultrual and Industrial Microorganisms, Budapest, Hungary, relates to a new microbial process for the preparation of pravastatin of formula (I)



(I)

from a compound of general formula (II),



(II)

wherein R^+ stands for an alkali metal or ammonium ion, by the submerged cultivation of a strain able to 6β-hydroxylate a compound of formula (II) by aerobic fermentation and by the separation and purification of the compound of formula (I) formed in the course of the bioconversion wherein the process comprises the steps of: a) cultivating a microorganism of the genus *Micromonospora* able to 6β-hydroxylate a compound of formula (II) – wherein R^+ is defined above – in a nutrient medium containing assimilable carbon and nitrogen sources and mineral salts at 25-32 °C, thereafter b) feeding the substrate until the end of bioconversion, c) fermenting the substrate until the end of bioconversion, then d) separating the compound of formula (I) from the culture broth and, if desired, purifying the same.

According to a yet more preferred embodiment, pravastatin is produced from either a wild strain or mutant strain of *Micromonospora* selected from the group consisting of *Micromonospora purpurea* IDR-P_4 [NCAIM P (B) 001271], *Micromonospora achinospora ssp. echinospora* IDR-P_6 [NCAIM P (B) 001272], *Micromonospora megalomicea ssp. nigra* IDR-P_6 [NCAIM P (B) 001273] and *Micromonospora rosaria* IDR-P_7 [NCAIM P (B) 001274]. According to the most preferred embodiment of the invention, pravastatin is produced with *Micromonospora* sp. IDR-P_3 [NCAIM P (B) 001268].

The present invention can be carried out by *in situ* fermentation, that is, by hydroxylation conducted in the presence of actively growing microorganisms using batch culture or fed-batch culture techniques.

The hydroxylation may be conducted by employing agitation, such as in shake-flask culture, or aeration and agitation in fermentors, when the compound of the formula (II) is added to the growing cultures. In such cases an anti-foaming agent may be employed.

The microorganisms may be cultivated and maintained using an appropriate nutrient medium containing carbon and nitrogen sources and inorganic salts and trace elements. Exemplary assimilable carbon sources include glucose, glycerol, dextrin, starch, ramnose, xylose, sucrose, soluble starch, etc. Exemplary assimilable nitrogen sources include soybean meal, corn steep liquor, pepton, yeast extract, meat extract, ammonium citrate, ammonium sulfate, etc. Inorganic salts such as calcium carbonate, sodium phosphates, potassium phosphates etc., may also be added to the culture medium. Preferred media for the growth of microorganisms are described in the examples.

Preferably the culture is an agitated liquid medium. The preferred temperature range for conducting the hydroxylation is from about 25°C to 37°C, most preferably about 25°C to 32°C. The preferred pH is from about 6.0 to 9.0, most preferably between about 7.0 to 8.5. The preferred shaking condition is about 200 rpm to 400 rpm, most preferably about 250 rpm.

Any compactin concentration can be used that will result in production of pravastatin. A compactin concentration of between about 0.1 and 10 g/liter, more preferably between about 0.3 and 3.0 g/liter, is well suited for *in situ* hydroxylation. The

percentage of conversion of compactin to pravastatin is not a critical feature of the inventive process. However, conversion preferably occurs to the extent of about 30% or more, preferably about 60% or more and yet more preferably about 90% or more.

The composition of the fermentation broth may be monitored by high performance liquid chromatographic method (HPLC) using conditions described in the Examples.

Pravastatin can be isolated from the fermentation broth by any method, e.g., extraction-reextraction, anion exchange chromatography or precipitation. The following isolation processes are well suited to isolating pravastin as a biosynthetic product of *Micromonospora*. However, these processes are provided for the sole purpose of completely disclosing the favored modes of obtaining pravastatin starting from compactin and a strain of the genus *Micromonospora* and are not intended to limit the invention in any way.

After finishing the bioconversion, pravastatin can be extracted either from the fermentation broth or from the filtrate obtained after the separation of the bacterium cells. Bacterium cells can be removed either by filtration or centrifugation. However, it is advantageous, especially in an industrial scale, to perform a whole broth extraction. Extraction solvents are any solvent that is not wholly miscible with water. Preferred extraction solvents have low solubility in water. Especially preferred solvents include acetic acid esters having a 2-4 carbon atom containing aliphatic alkoxyl moiety, such as ethyl acetate and isobutyl acetate.

In the course of our experiments it was recognized that pravastatin can be precipitated from an organic extract of the broth as a crystalline salt with secondary amines. Further, it was found that several secondary amines containing alkyl-, cycloalkyl-

, aralkyl- or aryl-substituents are especially well-suited for the salt formation. Among these, the following secondary amines are the most preferred, in part because of their low toxicity: dioctylamine, dicyclohexylamine and dibenzylamine.

The method of isolating the organic secondary amine salt of pravastin is illustrated with dibenzyl amine. Isolation of the dibenzylamine salt is carried out by adding dibenzylamine in 1.5 equivalent quantity related to the pravastatin content of the extract, then the extract is concentrated by vacuum distillation to 5% of its original volume, then another quantity of dibenzylamine is added into the concentrate in 0.2 equivalent ratio. The crystalline dibenzylamine salt is precipitated from the concentrate. The crystalline crude product is filtered and dried under vacuum, and is clarified with charcoal in methanol or acetone solution. Pravastatin dibenzylamine salt can be further purified by recrystallization from acetone.

Pravastatin organic secondary amine salts can be transformed to pravastatin with sodium hydroxide or sodium alkoxide. A preferred sodium alkoxide is sodium ethoxide.

The isolation of pravastatin via a secondary amine salt intermediate is a simpler procedure than any of the previously known isolation procedures. During the procedure, artifacts are not formed. Separation of pravastatin from by-products of the bioconversion and from the various metabolic products biosynthesized by the hydroxylating microorganism can be advantageously solved.

Another process for isolating pravastatin from the fermentation broth takes advantage of the fact that the bioconversion produces pravastatin in its acidic form. Thus, pravastatin can be isolated from the broth by adsorption on an anion exchange resin column, preferably from a filtrate of the broth. Strongly basic anion exchange resins like

a polystyrene-divinylbenzene polymer carrying quatemary ammonium active groups such as Dowex® Al 400 (OH^- form), Dowex® 1x2 (OH^- form), Dowex® 2x4 (OH^- form), Amberlite® IRA 900 (OH^- form) resins are well suited for absorbing pravastatin free acid from the broth. The material that absorbs on the ion exchange resin can be eluted from the column by aqueous acetic acid or a mixture of acetone and water containing sodium chloride. A 1% solution of sodium chloride in a (1:1) acetone:water mixture is a particularly preferred eluent. Pravastatin-containing fractions are combined and the acetone is distilled off under vacuum. The pH of the concentrate is adjusted with 15% sulphuric acid to a range of 3.5-4.0 and the acidified aqueous solution is extracted with ethyl acetate. Pravastatin can be re-extracted from the ethyl acetate extract using a 1/10 to 1/20 volume ratio of 5% sodium hydrogen carbonate or other mildly alkaline basic solution (pH 7.5-8.0).

Pravastatin can be recovered from the alkaline aqueous extract in a pure form by column chromatography on a non-ionic adsorption resin. In one method, any residual ethyl acetate that dissolved in the alkaline aqueous phase during extraction should be removed by vacuum distillation and then the aqueous extract is loaded on a Dialon HP-20 column. Pravastatin adsorbed on the column is purified by elution with aqueous acetone in which the acetone content is gradually increased, then the chromatographic fractions containing pravastatin as a single component are combined and concentrated under vacuum. The concentrate is clarified with charcoal and lyophilized. The pravastatin is then crystallized from an ethanol-ethyl acetate mixture, affording pravastatin in a quality acceptable for pharmaceutical application.

Another method for isolating pravastatin lactonizes pravastatin to improve separation from other acidic organic substances in the broth. Before extraction, the pH of either the fermentation broth or the filtrate of the broth is adjusted to 3.5-3.7 with a mineral acid, preferably with dilute sulphuric acid. The broth is then extracted with a water-immiscible organic solvent, preferably an acetic acid ester with a 2-4 carbon atom containing aliphatic alkoxyl moiety, such as ethyl acetate or isobutyl acetate. The ethyl acetate extract is washed with water and dried with anhydrous sodium sulphate. Then, pravastatin is converted to its lactone. The lactone ring closure may be carried out in dried ethyl acetate solution at room temperature under continuous stirring and using a catalytic amount of trifluoroacetic acid. Lactone ring closure can be monitored by thin layer chromatography ("TLC"). After the lactone has formed, the ethyl acetate solution is washed with 5% aqueous sodium hydrogen carbonate solution and then with water. The ethyl acetate solution is dried with anhydrous sodium sulphate and ethyl acetate is evaporated under vacuum. The residue is purified with silica gel column chromatography eluting with mixtures of ethyl acetate and hexane and gradually increasing the ethyl acetate content.

The purified pravastatin lactone is converted to pravastatin sodium by hydrolysis at room temperature in ethanol with an equivalent or more of sodium hydroxide. After the pravastatin sodium salt has formed, the pravastatin sodium can be precipitated with acetone. The precipitate is filtered and washed with acetone and *n*-hexane and dried under vacuum. The pravastatin sodium can be crystallized from an ethanol-ethyl acetate mixture to yield pravastatin sodium in a quality acceptable for pharmaceutical application.

Another method of isolating pravastatin uses chromatography on Sephadex LH-20 gel. Pravastatin exceeding the purity of 99.5% (measured by HPLC) can be produced by chromatography on Sephadex LH-20 gel.

Having thus described the invention with respect to certain preferred embodiments, the inventive processes for biosynthesis of pravastatin using *Micromonospora* and isolating pravastatin will further be illustrated with the following examples.

EXAMPLES

High performance liquid chromatography ("HPLC") was performed using equipment manufactured by Waters®. HPLC conditions: column packing Waters Novapack C_{18} 5μm reverse phase packing; UV detection: λ = 237 nm; injection volume: 10 μl; flow rate: 0.6-0.9 ml/min linear gradient; gradient elution: solvent A = acetonitrile-0.1M NaH_2PO_4 in water (25:75), solvent B = acetonitrile-water (pH 2 with H_3PO_4) (70:30). The gradient program is shown in Table 1.

Table 1

Time (min)	Flow rate (ml/min.)	Eluent A (%)	Eluent B (%)
0	0.6	100	0
2	0.7	100	0
20	0.9	0	100
21	0.9	0	100
22	0.9	100	0
27	0.7	100	0

Retention times: pravastatin (Na salt) 10.6 min; compactin (acid form) 19.5 min; pravastatin (lactone form) 17.3 min; compactin (lactone form) 23.5 min.

Example 1

A soluble starch agar medium ("SM", Table 2) was adjusted to a pH of 7.0 and then sterilized at 121°C for 25 minutes.

Table 2

Composition of SM medium	
Soluble starch	10.0 g
Yeast extract	5.0 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.25 g
KCl	0.2 g
$MgSO_4 \cdot 7H_2O$	0.2 g
Agar	15.0 g
Water	1000 ml

The SM medium was then innoculated with *Micromonospora sp.* IDR-P_3 [NCAIM P (B) 001268]. A spore suspension in distilled water (5 ml) was prepared from spores obtained from the 7-10 day old, soluble starch agar (SM) slant culture of *Micromonospora sp.* IDR-P_3 [NCAIM P (B) 001268].

The suspension was used to inoculate T1 inoculum medium (100 ml, Table 3) in a 500 ml Erlenmeyer flask after adjusting the pH of the T1 medium to 7.0 and sterilization at 121°C for 25 minutes.

Table 3

Composition of T1 medium	
Soluble starch	20.0 g
Yeast extract	10.0 g
$CaCO_3$	5.0 g
$CoCl_2 \cdot 6H_20$	2.0 mg
Water	1000 ml

The culture was shaken on a rotary shaker (250 r.p.m.; amplitude: 2.5 cm) for 3 days, at 32°C. Then, 5 ml portions of this inoculum culture were used to inoculate ten 500 ml Erlenmeyer flasks each containing TT medium (100 ml, Table 4) that had been adjusted to pH 7.0 and sterilized at 121°C for 25 minutes.

Table 4

Composition of TT medium	
Potato starch	30.0 g
Soybean meal	30.0 g
$CaCO_3$	5.0 g
$CoCl_2 \cdot 6H_20$	2.0 mg
Palm oil	2.0 g
Water	1000 ml

The bacteria were incubated at 32°C for 72 hours. The sodium salt of compactin (50 mg) was then added to each flask in distilled water, the bioconversion was continued at 32°C for a further 96 hours. The conversion of compactin sodium salt to pravastitin measured 82% by HPLC.

After finishing the fermentation, the cultures were combined. Pravastatin formed in an average concentration of 410 μg/ml. Pravastatin was isolated as follows. The fermentation broth was centrifuged at 2500 r.p.m. for 20 min. The supernatant of the broth and the cells of bacterium were separated. Water (250 ml) was added to the cells of bacterium and the suspension was stirred for one hour and filtered. The supernatant and filtrate were combined. The pH was adjusted to 4.0 with 15% sulphuric acid. The acidic filtrate/supernatant mixture was extracted with ethyl acetate (3x300 ml). The combined ethyl acetate extracts were washed with water (300 ml), dried with anhydrous sodium sulphate and concentrated under vacuum to 100 ml volume.

Pravastatin lactone was prepared from pravastatin by adding trifluoro acetic acid in catalytical amount at room temperature with continuous stirring. Formation of pravastitin lactone was monitored by TLC: adsorbent: Kleselgel (silica gel) 60 F_{254} DC (Merck) on aluminum foil backing; developing solvent: acetone:benzene:acetic acid (50:50:1.5) mixture; detection: phosphomolybdic acid reagent; R_f (pravastatin lactone) = 0.7. After lactonization was complete, the ethyl acetate was washed with 5% aqueous sodium hydrogen carbonate (2x20 ml), then water (20 ml), and dried with anhydrous sodium sulphate. Ethyl acetate was evaporated under vacuum. The residue (0.5 g) was separated by gradient column chromatography on 10 g of Kieselgel 60 adsorbent (column diameter: 1.2 cm) eluting with ethyl acetate-*n*-hexane mixtures of increasing polarity. Pravastatin lactone was eluted from the column with a mixture of 60% ethyl acetate/*n*-hexane. The fractions containing pravastatin lactone were combined and evaporated under vacuum. The residue (230 mg) was dissolved in ethanol (5 ml) and then 110 mole % of sodium hydroxide was added as a 1M ethanolic solution with stirring. Stirring was

continued for half an hour at room temperature. The solution was then concentrated to 2 ml volume. Acetone (4 ml) was added to the concentrate. The mixture was kept at +5°C overnight. The precipitate was filtered, washed with acetone (2 ml) and then *n*-hexane (2 ml) and dried under vacuum at room temperature. The resulting crude pravastatin was dissolved in ethanol. The solution was clarified with charcoal and then pravastatin (170 mg) was crystallized from ethanol-ethyl acetate mixture.

Characterization:

Melting point: 170-173°C (decomp.)

$[\alpha]_D^{20} = +156°$ (c=0,5, in water).

Ultraviolet absorption spectrum (20 μg/ml, in methanol): λ_{max} = 231, 237, 245 nm (log ε = 4.263; 4.311; 4.136).

Infrared absorption spectrum (KBr): ν OH 3415, ν CH 2965, ν C-0 1730, ν COO^- 1575 cm^{-1}.

^{1}H-NMR spectrum (D_2O, δ, ppm): 0.86, d, 3H (2-CH_3); 5.92, dd, J = 10.0 and 5.4 Hz, 1H (3-H); 5.99, d, J = 10.0 Hz, 1H (4-H); 5.52, br, 1H (5-H); 4.24, m, 1H (6-H); 5.34, br, 1H (8-H); 4.06, m, 1H (β-H), 3.65, m, 1H (δ-H); 1.05, d, 3H (2'-CH_3); 0.82, t, 3H (4'-H_3).

^{13}C-NMR spectrum (D_2O, δ, ppm): 15.3, q (2-CH_3); 139.5, d (C-3); 129.5, d (C-4); 138.1, s (C-4a); 127.7, d (C-5); 66.6, d (C-6); 70.1, d (C-8); 182.6, s (COO^-); 72.6, d (C-β); 73.0, d (C-δ); 182.0, s (C-1'); 18.8, q (2'-CH_3); 13.7, q (C-4').

Positive FAB mass spectrum (characteristic ions): 469 $[M+Na]^+$; 447 $[M+H]^+$.

Negative FAB mass spectrum (characteristic ions): 445 $[M-H]^-$; 423 $[M-Na]^-$;, *m/z* 101 [2-methyl-butyric acid-J].

Example 2

Bioconversion medium MT (Table 5) was adjusted to pH 7.0 and sterilized at 121°C for 25 minutes.

Table 5

Composition of MT Bioconversion Medium	
Potato starch	10.0 g
Dextrose	20.0 g
Soybean meal	10.0 g
Yeast extract	10.0 g
$CaCO_3$	5.0 g
$CoCl_2 \cdot 6H_20$	2.0 mg
Sunflower oil	2.0 g
Water	1000 ml

Ten 500 ml Erlenmeyer flasks each containing MT bioconversion medium (100 ml) were inoculated with the inoculum culture prepared in Example 1 and incubated at 28°C for 96 hours. The sodium salt of compactin (50 mg) was dissolved in a minimum of distilled water and added to each flask. Fermentation was continued for 72 hours. Then another 50 mg of compactin sodium salt in distilled water was added to each of the cultures and the fermentation was continued for another 72 hours.

The cultures were combined and pravastatin was isolated from the broth by the following procedure. The combined cultures, containing 750 mg of pravastatin according to the HPLC assay, were centrifuged at 2500 r.p.m. for 20 min. The separated cells of bacterium were stirred with water (250 ml) for an hour, then filtered. The supernatant and filtrate were combined and the pH of the resulting solution was adjusted to 3.5-4.0 with

15% sulphuric acid. The solution was extracted with ethyl acetate (3x300 ml). Then 150 mole% of dibenzylamine--calculated for the pravastatin content--was added to the ethyl acetate extract. The ethyl acetate extract was evaporated to about 30 ml volume and the suspension was kept overnight at 0-5°C. Precipitated pravastatin dibenzylammonium salt was filtered and washed on the filter with cold ethyl acetate and *n*-hexane and dried under vacuum. The crude pravastatin dibenzylammonium salt (1.1 g) was dissolved in acetone (33 ml) at 62-66°C. The solution was clarified with charcoal (0.1 g) for half an hour. The charcoal was removed by filtration from the solution and washed with warmed acetone (10 ml). Crystals precipitated from the concentrate and were dissolved again at 62-66°C. The solution was kept at +5°C overnight. The precipitate was filtered, washed with cold acetone and *n*-hexane and dried under vacuum. The pravastatin dibenzylammonium salt so obtained (0.7 g) was suspended in ethanol (10 ml), then 110 mole% of sodium hydroxide was added to the solution as a 1M aqueous solution. Stirring of the alkaline solution was continued for half an hour at room temperature. Water (30 ml) was added and the pH of the solution was neutralized. The ethanol was distilled off under vacuum. The resulting aqueous concentrate was separated by gradient column chromatography on a column filled with 50 ml of Diaion HP 20 resin (column diameter: 1.5 cm). The column was eluted with acetone-deionized water mixtures, increasing the concentration of the acetone in 5% increments. Pravastatin could be eluted from the column with a 15% acetone-deionized water mixture. Fractions were analysed by the TLC method given in the Example 1: R_f (pravastatin) = 0.5. Fractions containing pravastatin were combined and the acetone was evaporated under vacuum.

Lyophilization of the aqueous residue gave chromatographically pure pravastatin (390 mg).

Example 3

TT/2 medium (4.5 L, Table 6) was sterilized at 121 °C for 45 minutes in a laboratory fermentor and inoculated with the *Micromonospora sp.* IDR-P_3 inoculum shake culture in T1 medium (500 ml) prepared as described in Example 1.

Table 6

Composition of TT/2 Bioconversion Medium	
Glucose	75.0 g
Soluble starch	50.0 g
Soybean meal	50.0 g
Yeast extract	50.0 g
soya peptone	5.0 g
$CoCl_2 \cdot H_2O$	2.0 mg
$CaCO_3$	5.0 g
Water	1000 ml

The medium was then incubated at 28 °C, aerated with 150 L/h of sterile air and stirred with a flat blade stirrer at 300 r.p.m. The fermentation was continued for 72 hours and the sodium salt of compactin (2.5 g) was added to the culture. By the 48th hour of the bioconversion the compactin substrate was consumed from the fermentation broth. Additional compactin sodium salt (2.5 g) was added to the culture. The second dose of compactin substrate was consumed in 24 hours. The conversion rate of compactin sodium salt into pravastatin was 90%.

Example 4

TT/1 fermentation medium (4.5 L, Table 7) was adjusted to pH 7.0 and sterilized at 121°C for 45 minutes in a laboratory fermentor.

Table 7

Composition of TT/1 Bioconversion Medium	
Glucose	125.0 g
Potato starch	25.0 g
Soybean meal	50.0 g
Yeast extract (Gistex)	50.0 g
soya peptone	50.0 g
$CoCl_2 \cdot 6H_2O$	2.0 mg
$CaCO_3$	5.0 g
Sunflower oil	2.0 g
Water	1000 ml

The TT/1 medium was inoculated with the *Micromonospora sp.* IDR-P_3 inoculum shake culture (500 ml) prepared as described in Example 1. The culture was then incubated at 28°C, aerated with 200 L/h of sterile air and stirred with a flat blade stirrer at 400 r.p.m. for 96 hours. The sodium salt of compactin (2.5 g) was added to the culture as a sterile filtered aqueous solution. The fermentation was conducted at 28°C. By the fifth day of fermentation the compactin was consumed from the fermentation broth. Additional compactin sodium (7.5 g) was added in 2.5 g portions intermittently over two days. The additional compactin sodium salt was completely converted to pravastatin within four days of the first addition. At the end of the fermentation, compactin sodium salt (10 g) was converted to pravastatin (9 g, 90%).

Pravastatin at a concentration of 1800 µg/ml was isolated from the broth as follows. The culture broth (5 L) was centrifuged at 2500 r.p.m. for 20 min and the supernatant was separated from the cells of the bacterium. Water (2 L) was added to the separated cells and the resulting suspension was stirred for one hour and filtered. The supernatant and filtrate were combined and passed through a column containing Dowex® Al 400 (OH^-) resin (300 g, column diameter: 4 cm) at a flow rate of 500 ml/hour. The resin bed was washed with deionized water (1 L). The column was then eluted with a 1:1 acetone-water mixture (1 L) containing 10 g of sodium chloride, collecting in 50 ml fractions. The fractions were analyzed by the TLC method given in the Example 1. Fractions containing the product were combined and the acetone was distilled off under vacuum. The pH of the concentrate was adjusted to 3.5-4.0 value with 15% sulphuric acid. The concentrate was extracted with ethyl acetate (2x250 ml). Deionized water (40 ml) was added to the combined ethyl acetate extracts. The pH of the aqueous phase was adjusted to 7.5-8.0 with 1M sodium hydroxide. After 15 min stirring, the aqueous and ethyl acetate phases were separated. The aqueous alkaline extraction was twice repeated. The combined alkaline aqueous solutions were concentrated to 50 ml volume and the residue was separated by chromatography over Diaion® HP20 (Mitsubishi Co. Japan, 600 ml, column diameter 3.8 cm). The column was washed with deionized water (600 ml), then eluted with acetone-deionized water mixtures, increasing the concentration of acetone in the eluent in 5% increments. The eluent was collected in 50 ml fractions. The eluent was analysed by the TLC method given in the Example 1. Pravastatin was eluted from the column in the 15% acetone-deionized water mixture. Fractions containing pure pravastatin as determined by TLC were combined and the solution was concentrated

under vacuum to a volume of 150 ml. The concentrated eluent was clarified by stirring over charcoal (0.6 g) at room temperature for 1 hour. The charcoal was filtered off and the filtrate was lyophilized. The resulting lyophilised pravastatin (6.5 g) was crystallized twice from a mixture of ethanol and ethyl acetate. The precipitate was filtered and washed with ethyl acetate (20 ml) and *n*-hexane (20 ml), and dried under vacuum at room temperature to obtain chromatographically pure pravastatin (4.6 g).

Example 5

The sterile soluble starch medium SM of Example 1 was innoculated with *Micromonospora echinospora ssp. echinospora* IDR-P_5 [NCAIM P (B) 001272] bacterium strain and incubated for ten days. A spore suspension in distilled water (5 ml) was prepared from spores obtained from the ten day old soluble starch medium and the suspension was used to inoculate 100 ml of the sterile T1 inoculum medium described in Example 1 in a 500 ml Erlenmeyer flask. The culture was shaken on a rotary shaker (250 r.p.m., 2.5 cm amplitude) for 3 days at 28°C. Then, 5 ml portions of the obtained culture were transferred to ten 500 ml Erlenmeyer flasks, each containing 100 ml of bioconversion media TT/1 that had been sterilized by heating to 121°C for 25 min. The composition of the TT/1 medium is described in Example 3. Flasks were incubated with shaking on a rotary shaker (250 r.p.m., 2.5 cm amplitude) for 3 days at 25°C. Compactin sodium salt (10 mg) was added as a sterile filtered aqueous solution to each of the flasks. Fermentation was continued for 168 hours at 25°C. At the end of the bioconversion, the pravastatin content of the fermentation broth was 40 μg/ml as determined by HPLC.

Example 6

Inoculation, incubation, fermentation and substrate feeding were carried out with the *Micromonospore megalomicea ssp. nigra* IDR-P_6 [NCAIM P (B) 001273] bacterium strain as described in Example 5. The pravastatin content of the fermentation broth after 168 h was determined to be 50 µg/ml by HPLC.

Example 7

An inoculum culture of the *Micromonospora purpurea* IDR-P_4 [NCAIM P (B) 001271] bacteria strain (5 ml) was prepared according to the method described in Example 1. The inoculum culture was used to seed TT/14 medium (100 ml, Table 8) in 500 ml Erlenmeyer flasks after adjustment of the pH of the TT/14 medium to 7.0 and sterilization at 121 °C for 25 min.

Table 8

Composition of TT/14 Bioconversion Medium	
Potato starch	5.0 g
Glucose	25.0 g
Yeast extract (Gistex)	15.0 g
soya peptone	15.0 g
$CaCO_3$	5.0 g
$CoCl_2 \cdot 6H_20$	2.0 mg
Tap water	1000 ml

The flasks were shaken on a rotary shaker (250 r.p.m., 2.5 cm amplitude) for 3 days. Compactin sodium salt feeding, bioconversion and determination of the pravastatin content were carried out as described in Example 5. At the end of the bioconversion the pravastatin content of the fermentation broth was 40µg/ml, as measured by HPLC.

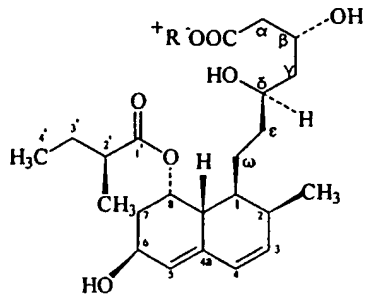
Example 8

Inoculation, incubation, fermentation and compactin sodium salt feeding were carried out with the *Micromonospora rosaria* IDR-P_7 [NCAIM P (B) 001274] bacterium strain following the method described in Example 1. At the end of the bioconversion, 350 µg/ml pravastatin was in the fermentation broth, as measured by HPLC.

Having thus described the invention with reference to certain preferred embodiments and with examples, those skilled in the art will appreciate variations that do not depart from the spirit and scope of the invention as described above and claimed hereafter.

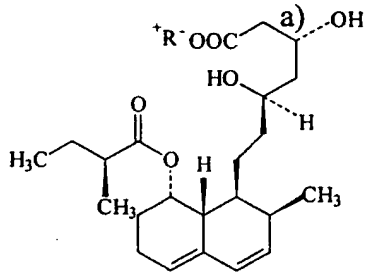
WE CLAIM:

1. A microbial process for the preparation of the compound of formula (I)



(I)

from a compound of the general formula (II)



(II)

wherein R^+ stands for an alkali metal or ammonium ion, by a strain able to 6β-hydroxylate a compound of formula (II) by fermentation and by the separation and purification of the compound of formula (I) formed in the course of the bioconversion comprising the steps of

a) cultivating a microorganism of the genus *Micromonospora* able to 6β-hydroxylate a compound of formula (II), wherein R^+ is as defined above,

on a nutrient medium containing assimilable carbon and nitrogen sources, thereafter

b) feeding a substrate to be transformed into the developed culture, then

c) fermenting the substrate until the end of bioconversion, then

d) separating the compound of formula (I) from the culture broth and, if desired, purifying the same.

2. The process of claim 9 wherein the microorganism is cultivated at a temperature of from about 25 to about 37°C.

3. The process of claim 10 wherein the microorganism is cultivated at a temperature of from about 25 to about 32°C.

4. The process of claim 9 wherein the nutrient medium is an aqueous liquid.

5. The process of claim 9 wherein the nutrient medium further comprises mineral salts.

6. The process as claimed in Claim 9 wherein the *Micromonospora sp.* IDR-P_3 strain deposited at the National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary under the number NCAIM P (B) 001268, or a mutant strain thereof, which is able to 6β-hydroxylate a compound of general formula (II) is cultivated.

7. The process as claimed in Claim 9 wherein the *Micromonospora purpurea* IDR-P_4 strain deposited at the National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary under the number NCAIM P (B) 001271, or a mutant strain thereof, which is able to 6β-hydroxylate a compound of general formula (II) is cultivated.

8. The process as claimed in Claim 9 wherein the *Micromonospora echinospora ssp. echinospora* IDR-P_5 strain deposited at the National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary under the number NCAIM P (B) 001272, or a mutant strain thereof, which is able to 6β-hydroxylate a compound of general formula (II) is cultivated.

9. The process as claimed in Claim 9 wherein the *Micromonospora megalomicea ssp. nigra* IDR-P_6 strain deposited at the National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary under the number NCAIM P (B) 001273, or a mutant strain thereof, which is able to 6β-hydroxylate a compound of general formula (II) is cultivated.

10. The process as claimed in Claim 9 wherein the *Micromonospora rosaria* IDR-P_7 strain deposited at the National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary under the number NCAIM P (B) 001274, or a mutant strain thereof, which is able to 6β-hydroxylate a compound of general formula (II) is cultivated.

11. The process as claimed in Claim 9 wherein R^+ is a sodium ion.

12. The process as claimed in Claims 9 to 18 wherein the compound of formula (I) formed during the fermentation is separated from the culture broth by adsorption on an anionic ion exchange resin or by extraction with a water-immiscible organic solvent, followed by the preparation of its lactone derivative or its secondary amine salt as an intermediate, or by purification of the alkaline aqueous extract obtained from the organic solvent extract of the fermentation broth with chromatography on a non-ionic adsorbing resin.

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(71) Applicant *(for all designated States except US)*: **IVAX CORPORATION** [US/US]; 4400 Biscayne Boulevard, Miami, FL 33137 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **JEKKEL, Antonia** [HU/HU]; Damjanich u. 38, H-1071 Budapest (HU). **AMBRUS, Gabor** [HU/HU]; Csalan u. 45/b, H-1025 Budapest (HU). **ILKOY, Eva** [HU/HU]; Munkacsy u. 37, H-1046 Budapest (HU). **HORVATH, Ildiko** [HU/HU]; Boloni Gy. u. 9, H-1021 Budapest (HU). **KONYA, Attila** [HU/HU]; Lovas I. u. 13, H-5000 Szolnok (HU). **SZABO, Istvan, Mihaly** [HU/HU]; Bertalan L. u. 11, H-1111 Budapest (HU). **NAGY, Zsuzsanna** [HU/HU]; Eotvos u. 12, H-1181 Budapest (HU). **HORVATH, Gyula** [HU/HU]; Kigyo u. 56, H-1052 Budapest (HU). **MOZES, Julianna** [HU/HU]; Borso u. 56, H-1173 Budapest (HU). **BARTA, Istvan** [HU/HU]; Kassai u. 10, H-1142 Budapest (HU). **SOMOGYI, Gyorgy** [HU/HU]; Jerney u. 70, H-1148 Budapest (HU). **SALAT, Janos** [HU/HU]; Veresegyhaz u. 21, H-1151 Budapest (HU). **BOROS, Sandor** [HU/HU]; Nyar u. 86, H-2134 Szod (HU).

(74) Agents: **BRAINARD, Charles, R.** et al.; Kenyon & Kenyon, One Broadway, New York, NY 10004 (US).

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Published:
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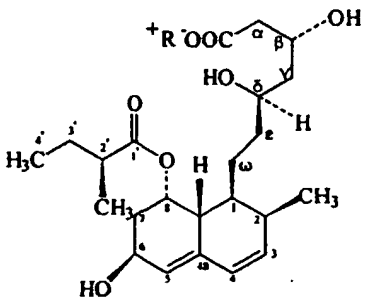
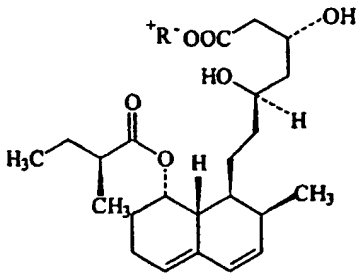
(54) Title: MICROBIAL PROCESS FOR PREPARING PRAVASTATIN



(I)



(II)

(57) Abstract: The present invention relates to a new microbial process for the preparation of the compound of formula (I) from a compound of general formula (II) wherein R^+ stands for an alkali metal or ammonium ion, by the submerged cultivation of the strain able to 6β-hydroxylate a compound of formula (II) in aerobic fermentation and by the separation and purification of the product of formula (I) formed in the course of the bioconversion, which comprises cultivating a strain of the genera *Micromonospora* able to 6β-hydroxylate a compound of the general formula (II), wherein R^+ is as defined above, on a nutrient medium containing assimilable carbon and nitrogen sources and mineral salts at 25-32 °C, thereafter feeding the substrate to be transformed into the developed culture, then fermenting the substrate until the end of bioconversion, then separating the compound of formula (I) from the culture broth and, if desired, purifying the same.

WO 01/03647 A3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/19384

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C07C 69/74; 61/12; 67/02
US CL :560/119, 256; 562/501
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 560/119, 256; 562/501

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN CAS File Registry, STRUCTURE, file medline; file biosis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Chem. abstr. Vol. 102 (Columbus, OH USA) abstract No. 130451, SANKYO CO., LTD., JAPAN "ML-236B Deriratives" JP 59175450 04 October 1984	1

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 27 NOVEMBER 2000	Date of mailing of the international search report 29 DEC 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PAUL J. KILLOS Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/19384

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 9-12
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claim 9 is dependent on itself and claim 12 is dependent on claims 8-18 but there are only 12 claims.

3. ☒ Claims Nos.: 2-12
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

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